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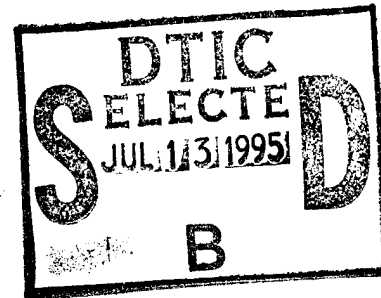
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BREAST CANCER

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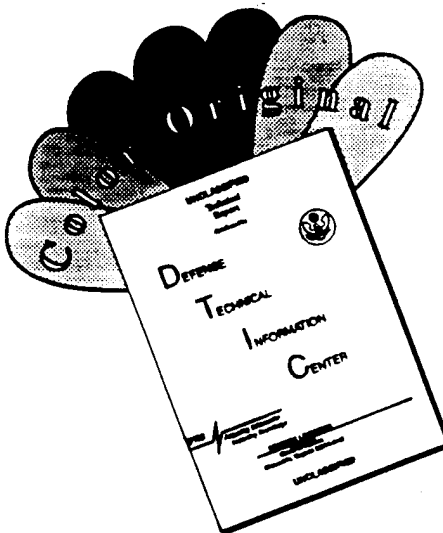
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13. ABSTRACT (Maximum 200 words) Sex hormone binding globulin (SHBG) is a high affinity binding protein for androgens and estrogens. This protein is normally produced in the liver, released into the blood and functions to regulate the amount of free androgen or estrogen available for action at target organs. Recently, receptors for SHBG have been identified on breast cancer cells, and on other steroid responsive tissues, including prostate carcinoma and endometrium. The goal of this series of experiments was to determine if mRNA for SHBG is expressed in breast cancer cell lines and tumor tissue. Two estrogen receptor positive cell lines were used, ZR-75-1 and MCF-7, and one estrogen receptor negative cell line, MDA-MB-231. SHBG mRNA was detected by Northern blot analysis in ZR-75-1 cells using a 500 bp 3' SHBG cDNA probe. Using the polymerase chain reaction (PCR), SHBG mRNA was detected in ZR-75-1, MCF-7 and MDA-MB-231 cells. In addition, SHBG protein production from these three breast cancer cell lines was detected by the method of immunoprecipitation using an affinity purified SHBG antibody. Also, evidence of alternative splicing of the SHBG mRNA in breast cancer cells was found. This was confirmed by DNA sequencing. In the alternate transcripts, exon 7 is deleted, accompanied by a point deletion in the beginning of exon 8 which results in a new stop codon and a shortened transcript. Amplification of RNA extracted from breast tissue by PCR revealed the presence of SHBG mRNA in breast tumor tissue and in tissue from women who have had cancer. SHBG mRNA was not detected in histologically normal tissue (reduction mammoplasty). In addition, 18 of 30 tumor samples analyzed did not contain detectable SHBG mRNA (by PCR). Thus, SHBG mRNA is expressed in some, but not all breast cancers. As was seen in the analysis of breast cancer cell lines, the presence of functional steroid receptors does not seem to be associated with SHBG mRNA expression.				
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Table of Contents

	Page
Front cover	1
SF 298	2
Foreword	3
Table of Contents	4
Introduction	5
Methods	10
Results (text)	14
Discussion	17
Summary	19
References	21
Figure legends	26
Figures	28

Introduction

Breast cancer is an economically and personally devastating disease for individuals and society. Breast cancer is now the second most common cancer in women with the risk for an individual woman to develop breast cancer over her lifetime at between 7 and 10%. The five year survival rate for breast cancer ranged between 40 and 64% depending on the population (1). In 1993, it was estimated that 182,000 new cases would be diagnosed (2). Some of the major risk factors for developing cancer include not only family history of breast cancer, but early menarche and late menopause, suggesting endocrine factors. While the 5 year survival rate for breast cancer has improved over time from 63% (1960) to around 75% (1981), the incidence of breast cancer remains high, at 86 cases (or greater) per 100,000 people per year (3). The estimated total cost for breast cancer in 1990 was \$3.8 billion with \$1.8 billion for medical care costs (4).

Role of Growth Factors and Oncogenes

The normal growth and development of breast tissue is regulated by estrogen with modulation by progesterone (5). The transformation of normal tissue to a cancerous tissue is now considered to be a multistage process which may involve gene activation, translocation, point mutation of growth factors or the loss of suppressor genes. While estrogen receptor is found in greater than 50% of breast cancers, only 60% of these respond to hormone manipulation. The concept has developed that steroid mediated growth of cancer cells involves locally produced (autocrine and paracrine) growth factors. A cascade of growth factors is necessary for the breast cell to begin abnormal growth, including platelet derived growth factor, transforming growth factor α , fibroblast growth factor, insulin like growth factors and possibly colony stimulating growth factor. These growth factors are classified as oncoproteins, and in cancerous breast tissue, the estrogen receptor itself may function as an oncoprotein (6).

Oncogenes and their expressed product, oncoproteins are associated with the abnormal growth of cancerous tissue. Generally, oncogenes are associated with the growth and differentiation of cells and tissues. Cancer, or the abnormal growth of cells, can result when these normally occurring genes, or proto-oncogenes, are expressed in an inappropriate time or place.

Generally oncogenes can be divided into five general classifications, growth factors, growth factor receptors, tyrosine kinases, G-proteins and nuclear proteins. The oncogenes identified in breast cancer tissue and cell lines include *c-myc*, which codes for a nuclear protein and often is associated with estrogen refractoriness (7, 8). Other nuclear protein oncogenes found in breast cancer include *c-myb* and *c-fos*. The G-protein oncogene *ras* p21 (9) is expressed in both benign and malignant tumors (10). A potential predictor of the biological behavior of breast cancer may be *C-erb-B-2*, (*HER2/neu*) (8)(8, 11), which has tyrosine kinase activity and is related to the intracellular domain of the epidermal growth factor (EGF) receptor. Breast cancer cells also can synthesize and secrete a number of growth factors, including transforming growth factor alpha and beta ($\text{TGF } \alpha/\beta$), insulin-like growth factor I and II (IGF I and II), and platelet derived growth factor (PDGF) (11). Consistent with the "multiple hit" theory of genetic deletions and rearrangements believed to be necessary for tumor formation, oncogenes have been identified in breast cancer cells comprising the cascade of cellular functions, from ligand, receptor, the intracellular component of the receptor to nuclear binding proteins, which modify and control cell growth. One class of oncogenes not discussed to this point are the tumor suppressor genes, of which *p53* and the retinoblastoma gene (*Rb1*) are examples.

Sex Hormone-Binding Globulin

Sex hormone-binding globulin (SHBG) may also function as an oncoprotein. SHBG is normally produced in the liver, and in its functional dimeric form, acts as a serum transport protein for androgens and estrogens (12, 13). Sites for the production of SHBG, and its related protein androgen binding protein (ABP), include liver (14), placenta (15) and testis (16). In addition to finding the full length mRNA for SHBG in liver and testis, alternatively processed transcripts have also been found in these tissues (17-19).. Up to this time, however, mRNA for SHBG had not been detected in a breast cancer cell line (MCF-7) (20). Interestingly, mRNA for SHBG has been found in prostate cancer cell lines (21), but not in normal prostate tissue. The SHBG mRNA isolated from prostate cancer cell lines ALVA-41, 101 and DU-145 exhibits a transcript at 1.6 kb, which is the size seen in Hep G2 cells and normal liver, and a larger transcript at 2.5 to 3 kb.

Many investigators have shown with immunocytochemistry that SHBG could be detected in human and primate prostate glands (22). Through the use of immunohistochemistry, SHBG reactive protein has been detected in human breast tissue (15, 23, 24) and in MCF-7 breast cells (22). Others have found that free as well as testosterone bound to SHBG could be taken up by the prostate by the single pass technique (25). When SHBG is added to cultures of ALVA-41 cells, the uptake of ^3H -thymidine increases, indicating that SHBG is able to stimulate DNA synthesis. Thus, SHBG may act as a paracrine or autocrine growth factor for these cells.

A separate growth promoting function for SHBG other than its transport function would at first glance appear to be novel. However, recently, a number of binding proteins including transferrin, ceruloplasmin and the IGF binding proteins have been shown to have functions other than those of ligand transport (26). SHBG contains sequence homology with only a few proteins. Other than androgen binding protein, only protein S (a vitamin K dependent clotting factor) has significant sequence homology (19, 27). The region with greatest homology is in the carboxy-terminal 100 amino acids, which has similar intron/exon structure, suggesting that the two proteins evolved from a common ancestral gene.

Alternate transcripts of SHBG message have been isolated from human liver and testis cDNA libraries (17, 18), and from rat testis, liver and brain cDNA libraries (28, 29). The alternate transcripts of SHBG mRNA from human and rat tissues are similar. Common features of the alternative transcripts are the use of alternate first exons and the splicing out of either exon 7 in the human transcript or exon 6 in the rat transcript. The preceding introns in each species, intron 6 in humans and intron 5 in the rat each contain repetitive Alu-like sequences, which may have a role in the processing of the transcribed RNA (18, 19). In the human alternative transcripts which include alternative first exons, exon 7 is spliced out and a frame shift occurs in exon 8, producing a truncated protein. Also, the predicted amino acid sequence of the alternative proteins lacks the two asparagine glycosylation sites which are located in exon 8 of the wild type protein. As the first exon is replaced in these alternative proteins, the glycosylated threonine in exon 1 also is missing. These changes from native SHBG in the alternative protein may produce a molecule with a

function other than steroid binding. In addition, in fetal rat liver, an additional cDNA was isolated that represented a fusion protein of SHBG and histidine decarboxylase (29). Investigators have speculated that these alternative proteins also have different promoter regions than wild type SHBG leading to tissue specific expression or possibly differential regulation within the same tissue (18, 28, 29).

The SHBG gene is located on the short arm of chromosome 17 (17p), close to the location of the tumor suppressor gene p53 (30). In one study, loss of heterozygosity and other types of gene rearrangements on 17p near the p53 locus have been detected in over 40% of the breast cancer cases studied (31, 32). In ovarian cancer, it has been found that the allelic loss of chromosome 17p extends beyond the locus of p53 at 17p13 to more internal regions at 17p12 and 17p11.2 (33). The genetic rearrangement of chromosome 17p which often occurs in breast cancer clearly offers the potential for modification of the SHBG gene (34).

SHBG Receptors

Recently, receptors for SHBG have been demonstrated on breast cancer cell lines (35-38) and on cell membranes prepared from breast tumor samples (39). Besides breast cancer cells, a receptor for SHBG also has been found on decidual endometrium (40), premenopausal endometrium (41), placental syncytiotrophoblast (42) and endometrial adenocarcinoma (37). SHBG receptors could not be detected on tissues that were not steroid responsive (43), including muscle and lymphocytes. The link between the presence of steroid responsiveness and the presence of a receptor for SHBG is further supported by the observation that an estrogen receptor negative breast cancer cell line, MDA-MB-231, did not have detectable SHBG receptors, and that ER negative breast tumor tissue also did not have detectable SHBG receptors (39). Recently, Nakhla et al have reported that estradiol causes the rapid accumulation of cAMP in human prostate cells in the presence of SHBG (44). The rapid production of a second messenger in response to SHBG and estradiol binding supports the hypothesis of a functional significance for the SHBG receptor on cells.

The physiological role of the SHBG receptor is still uncertain. Investigators report either

one binding site with a K_d of 10^{-8} (35, 36), others report two binding sites of 10^{-11} and another at 10^{-8} (38, 45). The binding site with the greater affinity also has a lower capacity than the lower affinity binding site. One proposed reason for these different observations is the choice of ligand used in the experiments. The studies that only reported one binding site used rabbit SHBG as the labeled ligand, while the studies reporting two binding sites utilized labeled human SHBG. While the amino acid sequence of the protein from these two species is similar, they differ in post translational modification. Both species contain asparagine linked oligosaccharide, however only the human SHBG contains threonine linked oligosaccharide. In other hormone-receptor systems, the glycosylation of the ligand can play a role in the activity of the ligand after binding to the receptor (46, 47).

Objectives of This Study

This project was initially composed of three objectives: 1) to determine if SHBG mRNA was produced by breast cancer cells and cell lines, 2) to determine if SHBG could act as a growth factor for breast cancer cell lines, and 3) to determine if SHBG receptors were present on breast cancer cells. The first objective was met by analyzing the RNA from three breast cancer cell lines, MCF-7, ZR-75-1 and MDA-MB-231 and also by extracting RNA from 77 breast samples ranging from tumor samples to reduction mammoplasty tissue. SHBG message was detected by northern analysis and the polymerase chain reaction using reverse transcriptase (rtPCR) from the breast cancer cell lines, and by rtPCR amplification of SHBG RNA from the tissue samples. An unexpected finding was the presence of differentially spliced transcripts of SHBG message as identified by PCR. The studies on whether SHBG can influence the growth of breast cancer cells is ongoing. Our goal was to determine if SHBG could act as a growth factor, either in the presence or absence of estrogen. To determine this, we chose to study two estrogen receptor positive cell lines, MCF-7 and ZR-75-1 for their responsiveness. To this date, we have been unable to reproduce the estrogen induced growth of these cell lines that has been reported by others (48-51). Details of our experiments are described below. Finally, the study of SHBG receptors on breast cancer cell lines and tumor tissue was not conducted, as this area of research has been

performed by others (35-39, 41, 45, 52) (please refer to the SHBG receptor section above). The time allowed for this portion of the project was used for DNA sequencing of the PCR products and to determine if SHBG protein was produced by breast cancer cells. Details of these experiments are presented in the methods section following. In our initial round of PCR experiments, we had the consistent finding of two amplified bands of SHBG. Two experiments were performed to characterize the unexpected SHBG band. In the initial experiment we used oligonucleotide probes that were specific for the SHBG exons in the amplified product. The results of this experiment were consistent with the hypothesis of alternative splicing of the SHBG mRNA. To definitively determine this, DNA sequencing was performed. To accomplish this goal, the products from the ZR-75-1 and MCF-7 PCR reaction were cloned, and clones containing the 500 bp band and the 300 bp band were sequenced using the Sanger di-deoxy sequencing method. In addition, a third experiment was added to this project, to determine if the cultured breast cancer cells produce SHBG protein. These changes in the objectives for the study were approved by Dr. H.A. Musallam by telephone conversation.

Methods

Cell culture Three human breast cancer cell lines, MCF-7 and ZR-75-1 and MDA-MB-231, were obtained from the American Type Culture Collection (Rockville, MD), and maintained in tissue culture (75 cm² flasks). MCF-7 and ZR-75-1 cells were chosen as they contain functional estrogen receptors, while the MDA-MB-231 cells were chosen due to their lack of a functional estrogen receptor. The MCF-7 cells are maintained in DMEM with 10% fetal bovine serum (FBS), the ZR-75-1 and MDA-MB-231 cells in RPMI with 10% FBS. Media for all cell lines was supplemented with Pen G (0.2 units/ml), Streptomycin (0.2 µg/ml) and L-glutamine (2 mM).

Extraction of RNA from cultured cells and Northern analysis RNA was extracted from adherent cells by the guanidinium thiocyanate method (53). Recovered RNA was resuspended in DEPC treated water, and recovery estimated by measuring the OD₂₆₀.

The mRNA for SHBG was detected in total RNA extracted from ZR-75-1 cells using

conventional Northern analysis. 25 µg of total RNA was electrophoresed on a 1.5% agarose gel, blotted by capillary transfer to a nylon membrane and probed with a ³²P labeled 3'SHBG cDNA probe (54). The blot was exposed to X-ray film for two weeks at -70°C.

Breast tissue specimens were obtained from the Department of Anatomical Pathology at Madigan Army Medical Center. All samples were excess to the diagnostic needs. Tissue was initially fixed in formalin, then placed in solution "D" (53), containing guanidinium and β-mercaptoethanol, and the tissue was minced with a tissue homogenizer. The extracted RNA was re-suspended in DEPC treated water and recovery estimated by measuring the OD₂₆₀.

Polymerase Chain Reaction (PCR) "Reverse" PCR was used to detect mRNA for SHBG in MCF-7, ZR-75-1, MDA-MB-231 cells and from RNA extracted from formalin fixed tumor tissue (GeneAmp RNA PCR kit, Perkin Elmer Cetus), using a GeneAmp PCR System 9600 (Perkin Elmer). RNA extracted from ZR-75-1 was used as a positive control, as SHBG mRNA could be detected in these cells by conventional Northern analysis. 500 ng total RNA was used for PCR amplification of SHBG. The primers used were: forward primer 5'ACTCAGGCAGAATTCAATCTC3' (nucleotides 628-647, as reported by Hammond et al, (54), reverse primer 5'CTTTAATGGGAAGCGTCAGT3' (antisense strand, nucleotides 1148-1129, (54)). The primers were synthesized using a Milligen Cyclone DNA synthesizer, and purified by butanol extraction (55). The reverse primer was used in the RNA reverse transcription step. These primers were projected to amplify the SHBG mRNA from base 556 to 1065 of the complete cDNA as reported by Hammond et al (54). The reverse transcriptase step was done at 42°C for 30 minutes, followed by 99°C for 5 min and cooling to 5°C for 5 min. A three temperature PCR program was used for amplification of DNA: 30 sec at 94°C, 30 sec at 56°C and 45 sec at 72°C for 35 cycles. Total volume in the reverse transcriptase reaction was 20 µl, and in the PCR amplification, 100 µl. Ten µl of PCR product was electrophoresed on a 4% agarose gel and bands were visualized by ethidium bromide stain. The gel was transferred using Southern's procedure, including denaturing the DNA in the gel, to a nylon membrane (magnagraph, MSI), and the nucleic acids UV crosslinked to the membrane. The PCR product

from the breast cancer cell lines were analyzed using the 3'cDNA SHBG probe, labeled with digoxigenin-11-dUTP using the random primer method, and chemiluminescent detection, using the Genius 1 chemiluminescent kit (Boehringer-Mannheim). Hybridization temperature was at 65°C, and washes were also performed at 65°C. In replicate experiments, bands could be detected with as little as 10 seconds of exposure of the membrane to the x-ray film.

One µg total RNA was used for PCR analysis of SHBG from the formalin fixed breast tissue, using the same primer set and PCR conditions as described above. The PCR product from formalin fixed tumor tissue was analyzed using the 3'SHBG cDNA as probe, labeled with [α -³²P]-dCTP by the random primer method, and exposure to x-ray film for up to 2 weeks at -70°C. As a control for RNA integrity, a second mRNA was amplified from the formalin fixed breast tissue, β -2 microglobulin (56). The expected PCR product size for the β -2 microglobulin was 200 bp.

Exon specific probes: To further investigate the identity of the RNA products amplified by PCR, specific oligonucleotide probes were prepared for SHBG exons 6 (5'CAG GCAGCAGGCTCAGGCCACCT3', nucleotides 709-731), 7 (5'GTCTTGAGCCA AGGG TCGAAGATG3', nucleotides 883-900) and 8 (5'CAAGGTCAGAGGCTGGATGTGGAC3', nucleotides 1045-1065). The specificity of the probes was verified by searching the GeneBank database, distributed by the National Library of Medicine, for other DNA or RNA sequences that were similar to these probes. As was done with the primers used above, probes were synthesized using a Milligen Cyclone DNA synthesizer, and purified by butanol extraction (55). Integrity of the oligonucleotides was verified by two methods, ion-exchange HPLC using a mono-P column (Pharmacia), and by capillary electrophoresis using a Quanta 4000 (Waters Chromatography) equipped with a µPAGE-5 (J&W Fisons) gel filled capillary (data not shown). The oligonucleotides were labeled with digoxigenin-11-dUTP using the 3'tailing reaction using a oligonucleotide tailing kit (Genius 6, Boehringer Mannheim). Three duplicate lanes of PCR product were prepared on a 1.5% agarose gel, transferred to a nylon membrane, and the membrane then cut into three identical strips. Each membrane strip was probed with one of the exon specific

probes. Membranes were hybridized at 55°C for 2.5 hr, washed twice at room temperature, and twice at 45°C. Bound probes were detected by chemiluminescence (Genius 1 kit).

DNA Sequencing: To confirm the composition of the PCR amplified RNA, PCR product from ZR-75-1 and MCF-7 cells was cloned using the TA Cloning System (Invitrogen). In contrast with kit directions, the PCR product was not diluted prior to ligation into the pCR™ vector. Transformed cells were plated onto LB agar plates treated with ampicillin and X-Gal. Plates were incubated at 37° C for 20 h. White colonies were selected for further analysis. Restriction digests of the PCR clones revealed that the desired PCR products of approximate sizes 300 bp and 500 bp had been cloned. The clones selected for further analysis were produced from ZR-75-1 PCR products. A plasmid mini-prep (57) was prepared for each of the PCR inserts. The plasmid preps were treated with DNase free RNase, followed by alkali denaturation.

DNA sequencing was accomplished using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical). Double stranded sequencing was accomplished using the primer set used for the original PCR amplification with incorporation of [α -³³P]-dATP (Dupont/NEN) and inclusion of Mn⁺⁺ buffer to emphasize bands close to the primers.. Electrophoresis was performed with a 6%, 60 cm acrylamide gel (IBI Base Runner 200), and sequence was analyzed using an IBI Gel Reader and MacVector software (Kodak Scientific Imaging Systems).

SHBG Protein Production by Cancer Cells: MCF-7, ZR-75-1 and MDA-MB-231 cells were grown to 80% confluence in 25 cm² flasks. Cells were incubated in methionine free media for 6 hours prior to the addition of ³⁵S methionine containing media (100 μ Ci/ml) . Cells were incubated at 37° C for 18 hours in the labeling media. After removal of the culture media, the cells were lysed and centrifuged at 100,000 x G to remove cellular organelles and debris. Proteins produced by the cells was incubated with an affinity purified SHBG antibody, prepared in a goat (gift of Dr. Philip Petra). The protein-antibody complex was precipitated with protein-G agarose (Pierce), which had been pretreated with 5% human serum albumin to reduce non-specific binding to the beads. Two controls were included in the immunoprecipitation, incubation of the cell lysate with normal goat serum followed by precipitation with protein-G coated beads, and treatment of

the cell lysate with the protein-G agarose beads only. The samples were electrophoresed on a 14% SDS PAGE gel, stained with coomassie blue, and treated with Enlightning™ (Dupont/NEN) to enhance detection of the ³⁵S labeled proteins. The gel was dried, and exposed to x-ray film.

Cell growth experiments: A series of experiments have been performed to characterize the effects on cell growth of different factors, including estradiol and tamoxifen, on breast cancer cell lines MCF-7 and ZR-75-1. The ultimate goal was to investigate whether SHBG could act as an independent growth factor, or interact with estrogen induced cell growth. Our first goal was to reproducibly demonstrate estrogen induced growth of the breast cancer cells, prior to performing the study with purified SHBG. To date we have not been able to demonstrate estrogen stimulated cell growth in breast cancer cell lines. Typically, 5000 to 10,000 cells are added to the wells of a 96 well microtiter plate. Cells are cultured with 1% charcoal striped fetal calf serum, using phenol-red free media. Test solutions of estrogen and tamoxifen ranging from 10⁻¹¹M to 10⁻⁵ M are added the next day. The growth experiments have been performed for periods of 3 to 14 days, with fresh test solutions and media daily. At the end of the experimental period, the number of cells in the wells are estimated using a Cell Titer 96 AQ non-radioactive cell proliferation assay (Promega). The color reaction is quantitated using a microtiter plate reader equipped with 580 nm and 690 nm filters.

Results

Northern Analysis: The mRNA for SHBG could be detected in the breast cancer cell line ZR-75-1 by conventional Northern analysis (figure 1). Bands at 1.8 kB and 3.3 kB were detected with the SHBG 3' cDNA probe, which is consistent with the sizes of bands observed by others for SHBG mRNA isolated from prostate cancer cell lines (58).

SHBG PCR: The polymerase chain reaction (PCR) was used to further investigate whether mRNA for SHBG could be found in breast cancer cell lines MCF-7, ZR-75-1 and MDA-MB-231. As shown in figure 2, bands were easily detectable in the PCR product from RNA isolated from MCF-7, ZR-75-1 and MDA-MB-231 cells. Two major bands were detected with the 3'cDNA probe, one at approximately 500 bp (the expected product) and another at

approximately 300 bp. The two major bands, at ≈ 500 bp and ≈ 300 bp, have been consistently amplified in all experiments using RNA isolated from these three cell lines.

SHBG Protein Expression: Not only do the three breast cancer cell lines express the mRNA for SHBG, they also transcribe the RNA for SHBG into protein (figure 3a and 3b). The Hep G2 cell line (figure 3a) was included in this experiment as a positive control, as SHBG protein can be detected in the conditioned media from these cells using a SHBG IRMA assay (Farmos). We could not detect SHBG in the conditioned media from the breast cancer cells using the IRMA assay (data not shown). For each cell line tested, lane A was protein G coated agarose beads without any added antibody and lane B was using normal goat serum and the protein G agarose beads. These two lanes identify the non-specifically precipitated proteins. Lane C contains cell lysates treated with the affinity purified SHBG antiserum, and one specific band appears in these lanes, at 45 kDa. A faint band appears in lane b in the ZR-75-1 experiment. However, as this band is lighter than lane c and this band does not appear in the lanes containing normal goat serum for the other cell lines, we believe that this is an artifact. SHBG purified from serum is a 90 kDa dimer which appears as a 45 kDa monomer on SDS gel electrophoresis. Thus, the SHBG produced by these breast cancer cells is similar in size to normal SHBG produced by the liver.

Exon Specific Probes: To further characterize the two major bands amplified by rtPCR, exon specific probes were used (figure 4). In this figure, the SHBG PCR products from the prostate cell line DU-145, and from ZR-75-1 are shown. Both the 500 bp and 300 bp bands are labeled with exon probes 6 and 8, however, only the 500 bp band is detected when the probe for exon 7 is used. Exon probe 8 also detects a smaller band at 100 bp. It is unknown if this band represents a SHBG-like product or resulted from non-specific PCR amplification. This portion of the study indicated that the 500 bp band contained exons 5 (site of the 5' PCR primer), 6, 7 and 8, and the 300 bp band contained exons 5, 6, and 8, but was missing at least part of exon 7. The 500 bp band is thus consistent with the normal SHBG mRNA, and the 300 bp band may represent an alternative transcript.

DNA Sequence Analysis: The composition of the PCR products was verified by

sequence analysis (figure 5a). The sequence of the ≈ 500 bp PCR product (figure 5b) is consistent with the sequence of SHBG, as reported by others (27, 54). The ≈ 300 bp product (figure 5c) was sequenced to within 20 to 30 bases of each primer, showing that this PCR product contains exon 6 and exon 8, and is missing exon 7, consistent with the oligo-probe experiment shown above. The new splice site between exon 6 and 8 also includes a single base deletion, resulting in a reading frame shift and a new stop codon. This alternative processing of this SHBG mRNA is consistent with the pattern of alternative processing observed in testis transcripts of androgen binding protein (17, 18), and SHBG cDNAs found in liver extracts (17).

SHBG in Breast Biopsy Samples: To date, RNA has been isolated from breast tissue collected from 77 different patients. In all of these samples, PCR amplification of the control RNA, β -2 microglobulin was used to determine the integrity of the RNA in the sample was amplified. If the expected PCR product from the control RNA was not produced, the sample was classified as a non-interpretable sample. The tissue samples thus became stratified into four groups, SHBG and β 2 positive (group 1), SHBG negative and β 2 positive (group 2), SHBG positive and β 2 negative (group 3), and SHBG negative and β 2 negative (group 4). Of the 77 samples collected, analysis is not complete of 21 of them. They will be complete in the next 60 days. Eleven of the samples have been found to be positive for SHBG and β 2, and thus are true positives. Nineteen of the samples are SHBG negative and β 2 positive, and thus are true negatives. Of interest are the 5 samples that are SHBG positive, but are β 2 negative, and thus must be considered to be false positives. However, there have been recent reports that β 2 microglobulin may be modified in some cancers (59-61). If the site of the modification of the of the β 2 RNA is at one of the PCR primer sites, or the gene for β 2 is no longer transcribed, these samples would be recorded as β 2 negative, and the interpretation given that the RNA is of poor quality. To help resolve this question, a second control RNA and PCR reaction has been developed using a universally expressed enzyme, porphobilinogen deaminase, which was reported to be a useful positive control for RNA analysis from formalin-fixed tissues (62). As β 2 microglobulin is a component of the histocompatibility recognition complex of cell surface proteins

a change in the expression of this protein may alter the immune recognition of the cell, and potentially provide a survival benefit to an affected cancer cell.

SHBG and Cell Growth: Our efforts to characterize the effects on cell growth of SHBG have not progressed as planned. As shown in figure 6a, we cannot detect estrogen induced growth in either the MCF-7 or ZR-75-1 cells. Both estrogen and tamoxifen are toxic at the highest levels used in these experiments. This pattern of response is consistent between short treatment times (3 days) and longer treatment experiments (14 days). One possibility that we explored was whether the cells that we were culturing had functional estrogen receptors. The presence of estrogen receptors in MCF-7 and ZR-75-1 cells was confirmed using an estrogen receptor assay kit (New England Nuclear). We can detect an increase in cell number, as when the cells are cultured in serum free conditions using phenol red-free media, cell number does not change, with the addition of 1% charcoal stripped fetal calf serum, the cells steadily grow over a 14 day period (figure 6b). Our inability to demonstrate estrogen induced growth in MCF-7 cells apparently is not unusual (63). Future experiments will include strategies such as gradually weaning the cultured cells from fetal calf serum, different methods for preparing the steroid treatments, and use of different concentrations of fetal calf serum in the cultures.

Discussion

This series of experiments documents the expression of mRNA for SHBG in breast cancer cell lines and tumor tissue. SHBG messenger RNA was detectable by Northern analysis from ZR-75-1 cells. RNA species at 1.8 kB and 3.3 kB were detected which is similar to the sizes of SHBG mRNA detected from prostate cancer cell lines (21). This size distribution of RNA for SHBG in breast cancer cells, however is different from what is detected in a hepatoma cell line, Hep G2. In this cell line one transcript for SHBG is found, at 1.6 kB. The larger band at 3.3 kB from breast cells may represent incompletely processed mRNA for SHBG, as the predicted size for the SHBG message with the introns in place is approximately 3.3 kB to 3.5 kB (18).

Expression of SHBG messenger RNA was found in the three breast cancer cell lines studied, MCF-7, ZR-75-1 and MDA-MB -231. Studies in prostate cancer cell lines had indicated

that the level SHBG mRNA expression was regulated by steroid hormones, leading to the hypothesis that the presence of functional steroid hormone receptors was important for SHBG mRNA expression. This does not appear to be the case as MDA-MB-231 cells do not contain functional estrogen receptors, but still express SHBG mRNA at levels similar to the two estrogen receptor positive cell lines MCF-7 and ZR-75-1.

In addition to detection of the mRNA for SHBG in these breast cancer cells, we have also detected expressed SHBG protein from these three cell lines. The expressed SHBG from the breast cancer cells is similar in size to the SHBG produced by a liver cancer cell line and to protein that is purified from human serum (64). The similar size of breast origin SHBG to SHBG produced by Hep G2 cells and serum SHBG indicates that post translational processing of the protein in breast cells, including addition of carbohydrate chains, occurs. Thus, the SHBG produced by breast cancer cells may be able to bind to SHBG receptors on breast cancer cells and potentially trigger intracellular events in the cells, as was recently reported in the interaction of SHBG and prostate cells (44).

Two major PCR products are routinely amplified from the ZR-75-1, MCF-7 and MBA-MB-231 breast cancer cells. These different sized products are consistent with alternative splicing of the SHBG mRNA in these cells. The alternative processing of the SHBG mRNA was confirmed by experiments using exon specific probes and ultimately by sequence analysis. Alternative splicing of SHBG and its testis analog, androgen binding protein, has been reported in other tissues, including fetal rat liver (29), human testis (18) and human liver (17). The potential protein product of the alternative transcript of SHBG found in breast cancer cells may have a different function from SHBG as the deleted region contains an alternating leucine sequence that has been postulated to be a portion of the steroid binding domain (65). The alternating leucine segment of this portion of the protein also has been postulated to interact together to form a β -sheet which could contribute to the stability of the SHBG dimer (64). The loss of this sequence could mean that a protein product of an alternate transcript could exist as a monomer without steroid binding activity. Common features of the alternative transcripts of SHBG described in liver and

testis besides exclusion of exon 7 include and substitution of an alternative exon 1 at the 5' end of the message. It remains to be determined if similar alternative transcripts of SHBG are found in breast cancer cells.

In addition to identifying SHBG RNA in breast cancer cell lines, we also have detected, through the use of PCR, SHBG messenger RNA in 11 of 30 formalin fixed breast tissue samples in which the analysis of the control RNA, β -2 microglobulin was positive. One of these samples is from a prophylactic mastectomy (right breast) in a patient with a history of infiltrating lobular carcinoma in her other breast. The right breast had fibrocystic changes including florid ductal hyperplasia, but no neoplasia was identified. The other breast tissue samples that tested positive for SHBG mRNA expression were a mix of ductal carcinoma and lobular carcinoma. The positive samples included both estrogen and progesterone positive and negative tumors, and cases with node involvement and cases with no axillary node metastasis. To date, 19 breast samples have tested negative for SHBG mRNA. These samples include infiltrating ductal carcinomas, fibroadenoma, and lobular carcinoma. These tumor samples also include both estrogen and progesterone receptor positive and negative samples and cases in which lymph nodes are and are not involved. This group also includes the reduction mammoplasty samples, which are histologically normal, and do not have detectable expression of SHBG mRNA. Thus, expression of SHBG mRNA in breast tissue samples only has been detected in tumor samples, or from women who have had breast cancer.

Summary

We have shown that SHBG mRNA is expressed in breast cancer cell lines and in tumor tissue. In contrast to the need for a functional estrogen receptor for the SHBG receptor to be present, SHBG mRNA is expressed in steroid receptor positive and negative breast cell lines and tumors. In addition, we have shown that in breast cancer cells, the mRNA for SHBG is transcribed into SHBG protein. To date, approximately 36% of the breast tissue samples studied express SHBG mRNA. Normal breast tissue from reduction mammoplasty does not contain detectable SHBG mRNA, indicating that the presence of SHBG mRNA is a characteristic of

abnormal breast tissue. Not all breast cancers express SHBG mRNA, and the differences between SHBG positive and negative tumors will be the subject of further investigation.

We have partially characterized alternate transcripts of SHBG produced in breast cancer. We have shown that exon 7 and one base from the start of exon 8 is removed from the normal SHBG message. This pattern of alternate mRNA processing has been described in other tissues, but not in breast. It is predicted that the alternate protein may be a monomer, and lack steroid binding activity. Future plans include production of full length cDNAs for SHBG mRNAs expressed in breast cancer cells, characterize the 5' end on the mRNAs and express these cDNAs and use in-vitro methods to begin to characterize the effects of the alternate proteins on breast cancer cells.

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Figure legends:

Figure 1: a) Northern blot analysis of expression of SHBG mRNA from ZR-75-1 breast cancer cells. Blot was probed with a ^{32}P 500 bp 3'cDNA for SHBG. Two bands were detected with this probe, at 3.3 kB and 1.8 kB.

Figure 2: Amplification of SHBG mRNA by reverse PCR. 500 ng of RNA from ZR-75-1, MCF-7 and MDA-MB-231 breast cancer cells was used. 10 μl of the PCR product (100 μl total volume) was electrophoresed on a 4% agarose gel, transferred to a nylon membrane and probed with the 3'cDNA probe labeled for chemiluminescent detection. Two bands are routinely amplified, at $\approx 300\text{bp}$ and $\approx 500\text{bp}$. Sizes of the amplified products are indicated.

Figure 3: Immunoprecipitation of SHBG from breast cancer cell lines. Figure 3a includes HEP G2 and MDA-MB-231 cell lysates, figure 3b contains MCF-7 and ZR-75-1 cell lysates. An affinity purified SHBG antibody (raised in a goat) was used to immunoprecipitate SHBG produced in MDA-MB-231, MCF-7 and ZR-75-1 breast cancer cells. Hep G2 cell lysate was included as a positive control. For each cell lysate tested, lane A is treatment with protein G coated agarose beads alone, lane B is treatment with normal goat serum and the protein G agarose, and lane C is treatment with the affinity purified goat anti-SHBG and protein G agarose. For each cell line, an SHBG specific band, at 45 kDa, is present in lane C.

Figure 4: Probes specific for exons 6, 7 and 8 were used to initially characterize the PCR product. Shown in this figure is PCR product from DU-145 (a prostate cancer cell line) and ZR-75-1. The probe for exons 6 detected the 500bp and 300 bp bands, while the exon 8 probe detected an additional band at 100 bp. The exon 8 probe signal is faint in the ZR-75-1 cells. The probe for exon 7 only detects the $\approx 500\text{ bp}$ band. This indicated that the $\approx 300\text{ bp}$ band may be missing exon 7.

Figure 5: A) Two clones were sequenced, one containing a ≈ 500 bp insert and the other a ≈ 300 bp insert. Both strands of the DNA were sequenced. On the auto radiogram, it can be seen where the sequences of the two samples diverge (indicated by arrows).

B) The partial DNA sequence of the ≈ 500 bp PCR fragment is identical to the reported DNA sequence. The bases are numbered to be consistent with the sequence as reported by Hammond et al.(54)

C) The partial DNA sequence of the ≈ 300 bp PCR product confirmed that exon 7 is missing from this band. Also a single base deletion has occurred at the beginning of exon 8, potentially resulting in a frame shift and the introduction of a new stop codon. This pattern of alternative splicing of SHBG, and its related protein ABP, involving the deletion of exon 7 and a single base deletion has been reported in RNA extracted from testis (17, 18) and liver (17).

Figure 6 A) Cultures of MCF-7 and ZR-75-1 cells were treated with differing concentrations of estradiol and tamoxifen for three days. The only effect of the treatments was a toxic effect on the cells at the highest doses used. All other wells grew at the same rate as the treatment blank.

B) MCF-7 cells were cultured either in serum free conditions, or with the addition of 1% charcoal striped fetal calf serum in the presence or absence of estradiol. Cells did not appear to grow in the serum free conditions, however the addition of estrogen appeared to slow growth in the presence of 1% charcoal striped fetal calf serum.

Figure 1

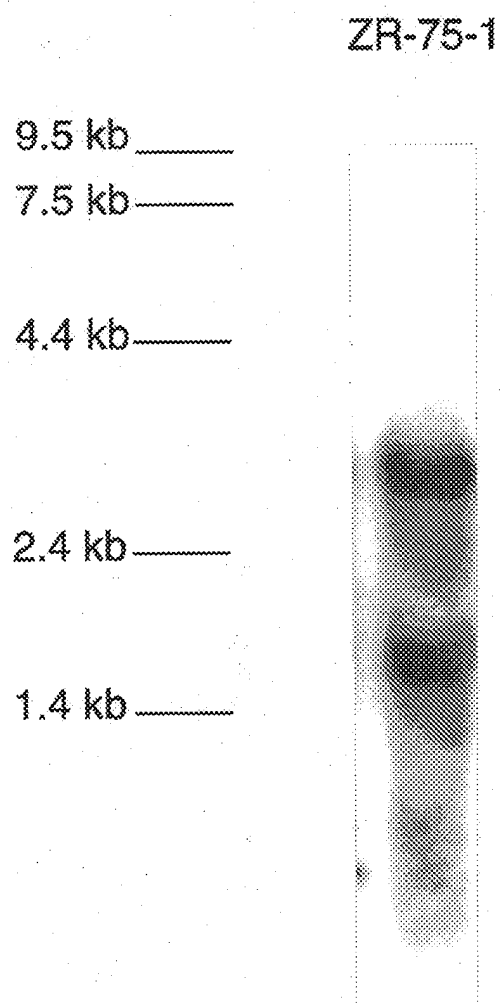


Figure 2

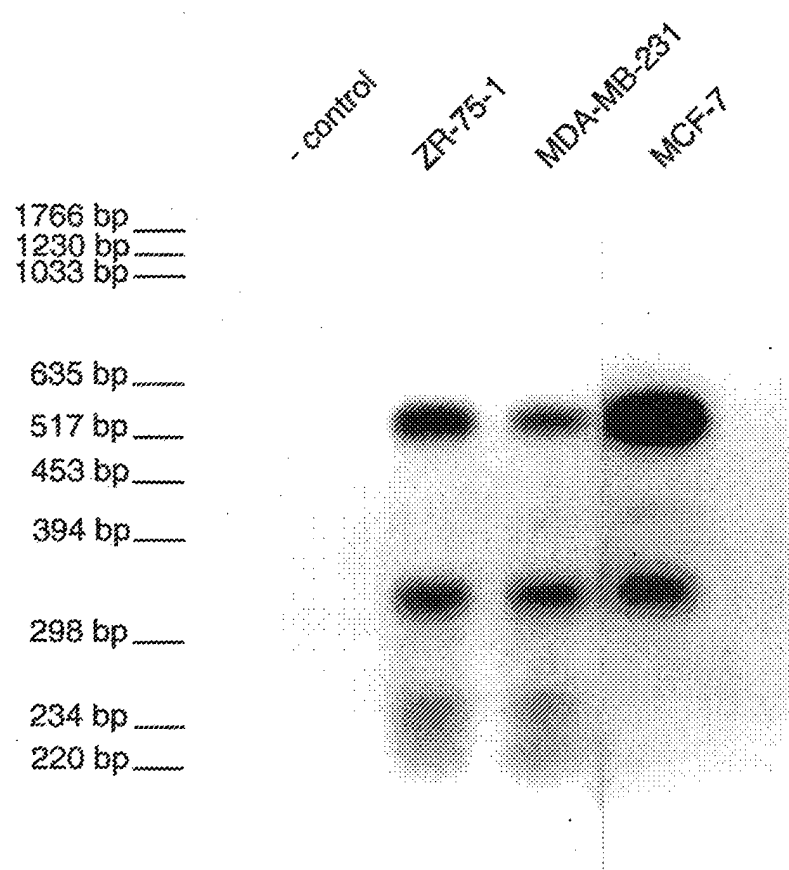


Figure 3a

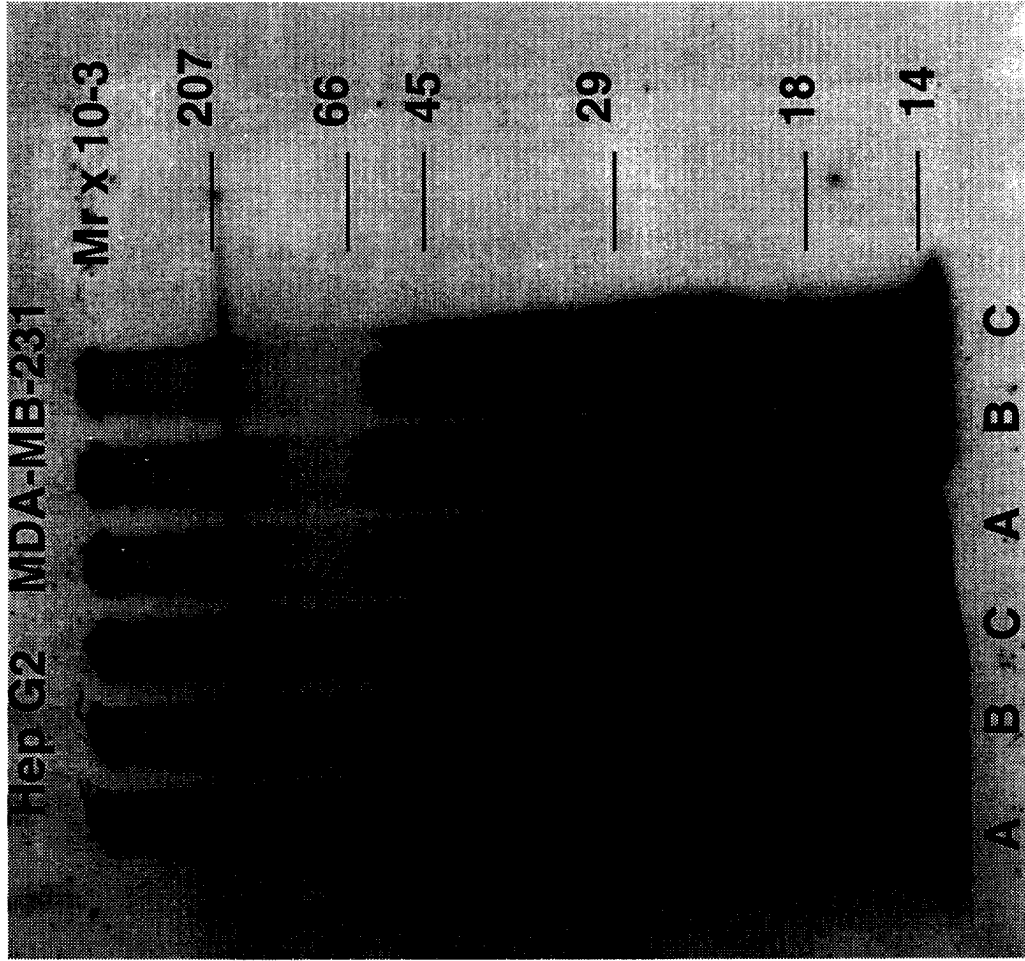


Figure 3b

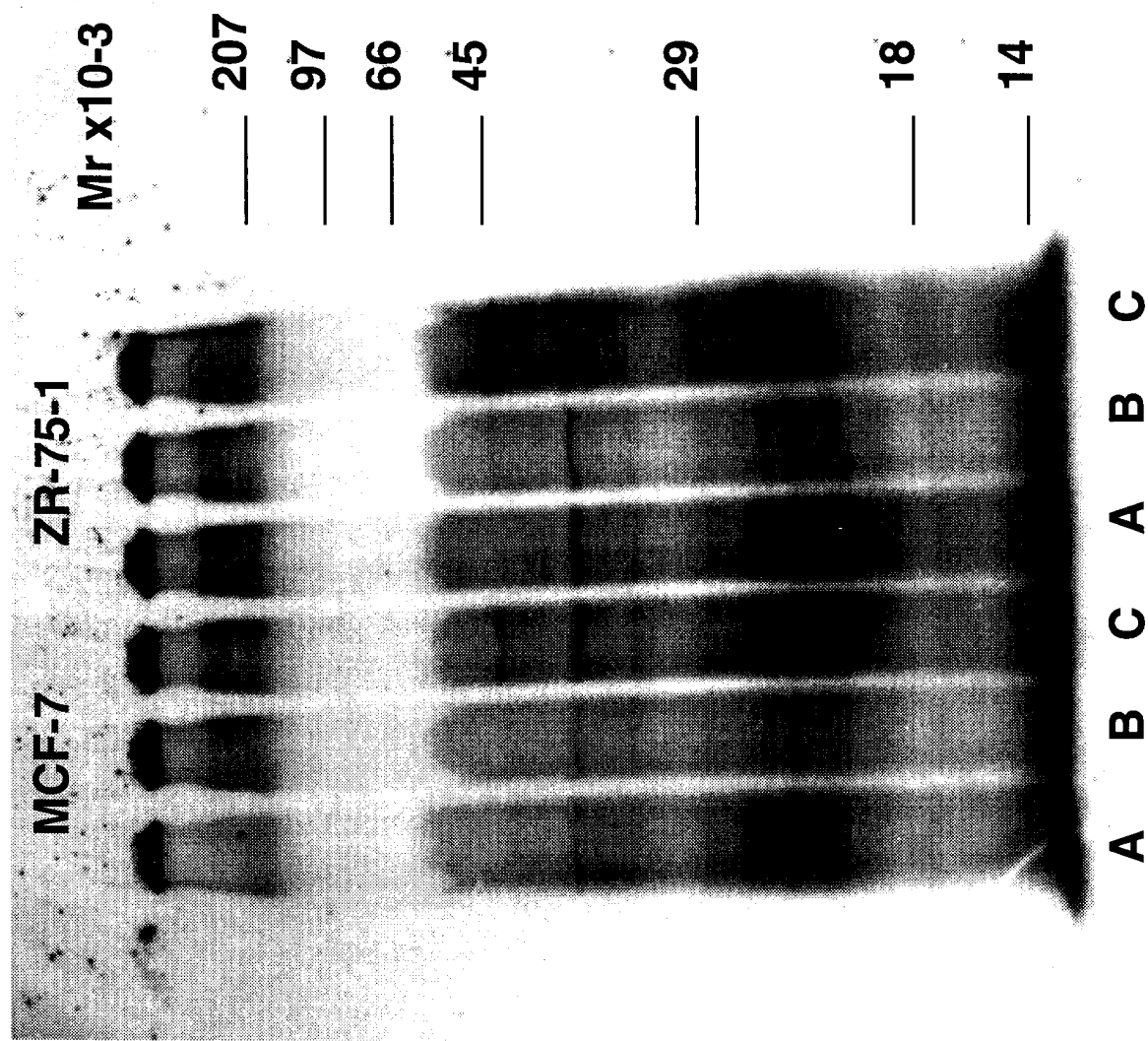


Figure 4

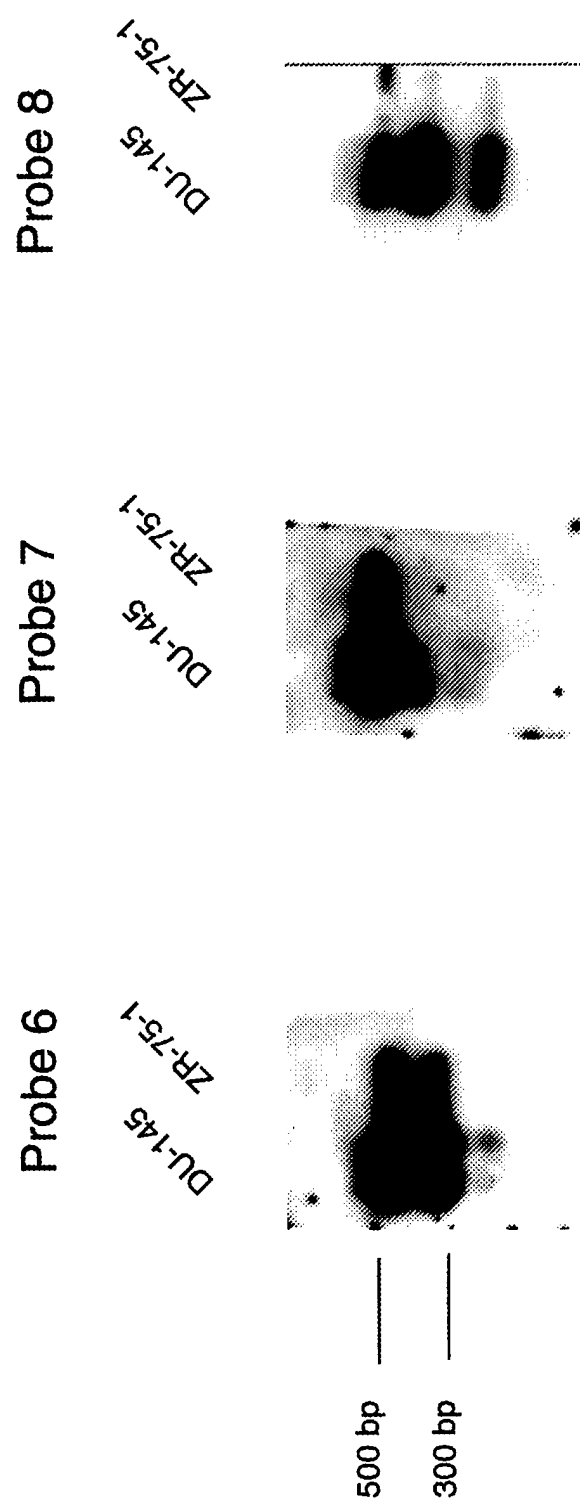


Figure 5a

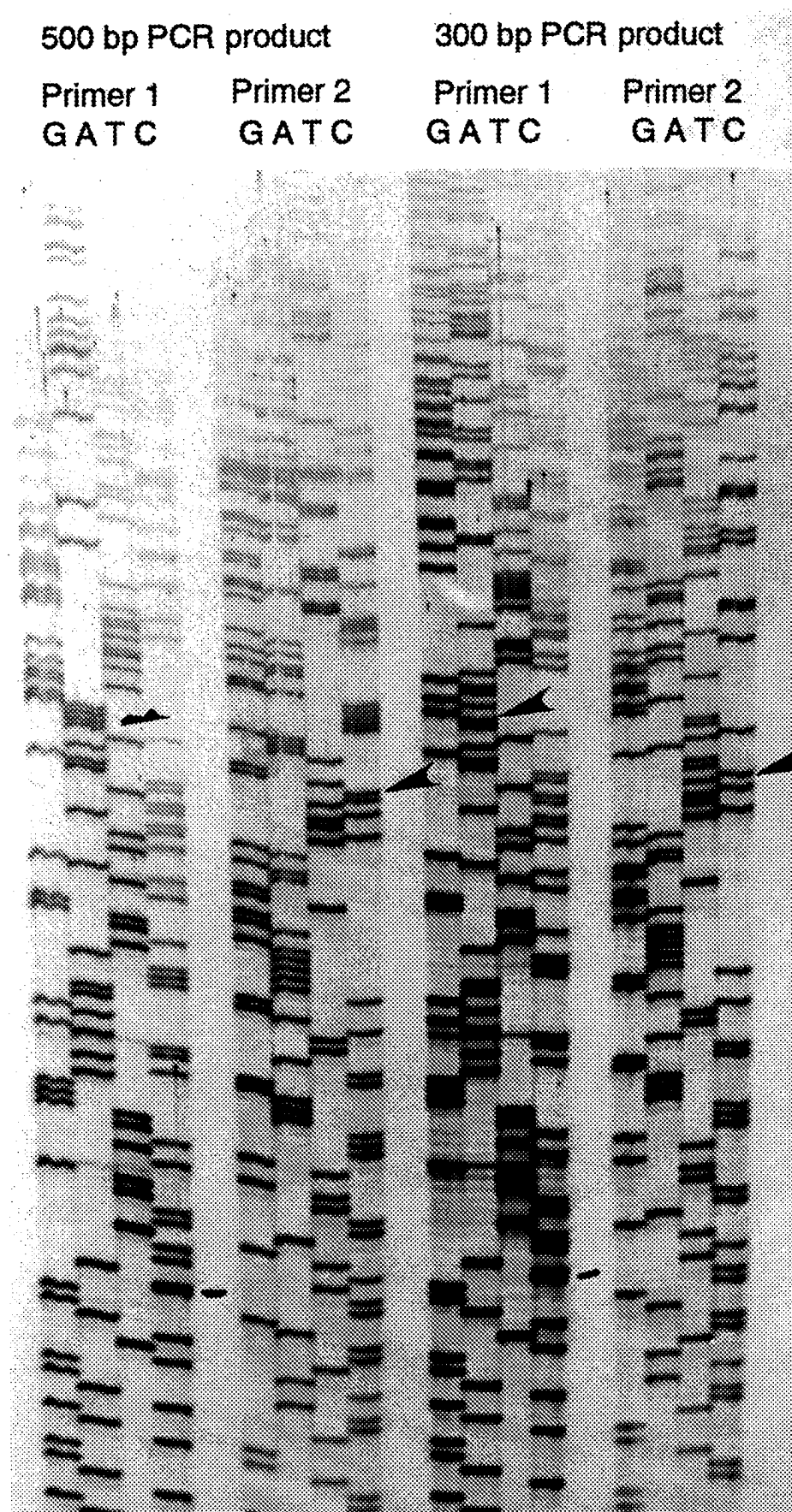


Figure 5b

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      665                      685                      705
      *                        *                        *
G CCT CAT GCA GAG CCC TGG GCC TTC TCT TTG GAC CTG GGA CTC AAG
<--Exon 6

                      725                      745
                      *                        *
CAG GCA GCA GGC TCA GGC CAC CTC CTT GCT CTT GGG ACA CCA GAG

          765                      785
          *                        *
AAC CCA TCT TGG CTC AGT CTC CAC CTC CAA GAT CAA | AAG GTG GTG
                      Exon 6 end ---> | <---Exon 7 start

          805                      825
          *                        *
TTG TCT TCT GGG TCG GGG CCA GGG CTG GAT CTG CCC CTG GTC TTG

      845                      865                      885
      *                        *                        *
GGA CTC CCT CCT CAG CTG AAG CTG AGT ATG TCC AGG GTG GTC TTG

          905                      960
          *                        *
AGC CAA GGG TCG AAG ATG AAG GC      //      GCC AAG CTT

          980                      1000
          *                        *
CAA GGG CGT CTC TTC CTG GGG GCT TTA CCA | GGA GAA GAC TCT TCC
                      Exon 7 end ---> | <---Exon 8 start

          1020                      1040
          *                        *
ACC TCT TTT TGC CTG AAT GGC CTT TGG GCA CAA GGT CAG AGG CTG

      1060                      1080                      1100
      *                        *                        *
GAT GTC GAC CAG GCC CTG AAC AGA AGC CTG GAG ATC TGG ACT CAC

AGC TG
Exon 8 --->

```

Partial sequence of 500 bp PCR product. Numbers above the sequence refer to the complete cDNA sequence as reported by Hammond et al. (53)

Figure 5c

```

      665                      685                      705
      *                        *                        *
G CCT CGT GCA GAG CCC TGG GCC TTC TCT TTG GAC CTG GGA CTC AAG
<---Exon 6

              725                      745
              *                        *
CAG GCA GCA GGC TCA GGC CAC CTC CTT GCT CTT GGG ACA CCA GAG

          765                      785      |      1000
          *                        *                        *
AAC CCA TCT TGG CTC AGT CTC CAC CTC CAA GAT CAA | GAG AAG ACT
                                Exon 6 end--->|<---Exon 8 start
                    1020                      1040
                    *                        *
CTT CCA CCT CTT TTT GCC TGA ATG GCC TTT GGG CAC AAG GTC AGA

          1060                      1080
          *                        *
GGC TGG ATG TGG ACC AGG CCC TGA ACA GAA GCC ATG AGA TCT GGA

      1100
      *
CTC ACA GCT
Exon 8 --->

```

Partial sequence of the 300 bp PCR product. Numbers above the sequence refer to the complete cDNA sequence as reported by Hammond et al. (53)

Figure 6a

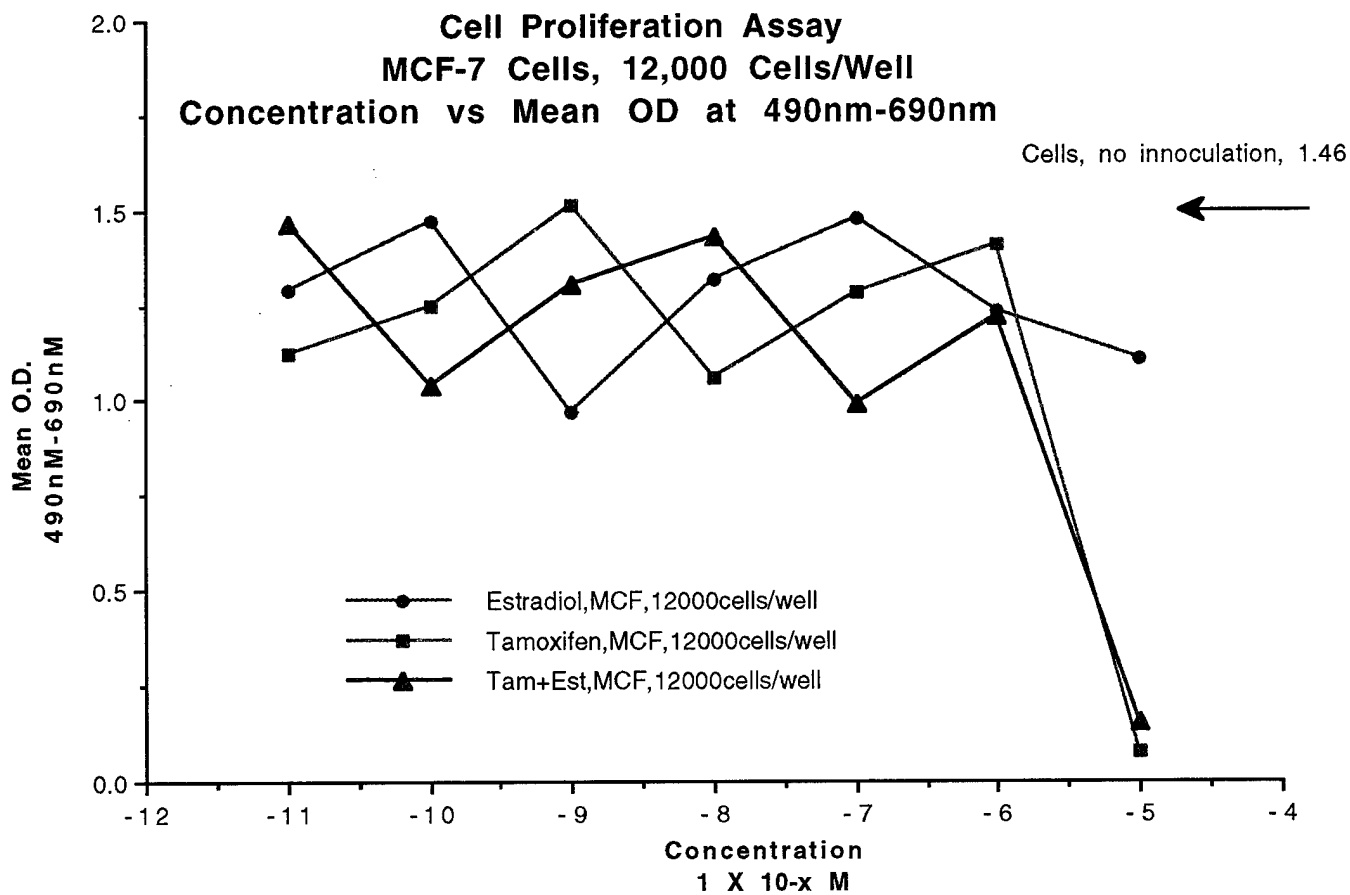
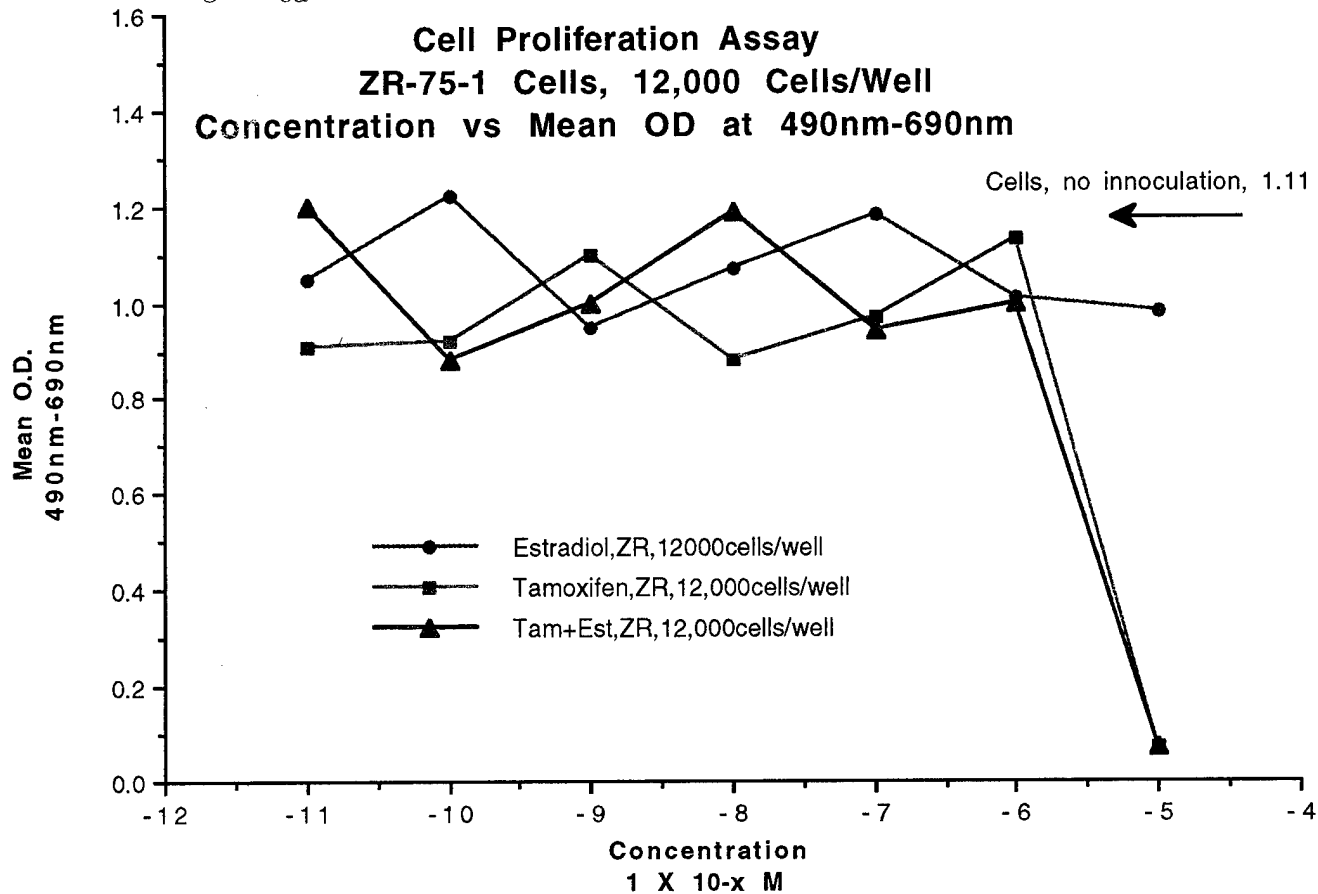


Figure 6b

